used, due consideration must also be paid to the costs of all stages of the separation procedure. Such consideration should cover not only the basic costs of the chemicals employed, but also all costs associated with disposal and/or recycling of those chemicals after use. Some affinity procedures may involve specific eluents (e.g. enzyme co-factors) whose expense is greater than that of the simple strategies of changes in pH, ionic strength or dielectric constant used to elute many adsorbates. These economic considerations become of much greater importance in the design of process-scale procedures and are often overlooked at the laboratory scale.

Conclusions and Future Prospects

There are no technical barriers preventing the use of affinity separations in the production of biological molecules and entities. The biochemical engineering principles associated with the scale-up and optimization of affinity separations are well developed and the resultant conclusions are readily implemented. The conservatism surrounding their current use stems from the widespread lack of suitable affinity ligands. It is anticipated that novel molecules emerging from new techniques such as phage display technology and combinatorial chemistry will be excellent candidates for use of ligands in affinity separations. Affinity separations will be used in the purification of soluble biomolecules and also in the isolation of more complex species such as viruses, cells and other products for use in gene therapy. Affinity separations will therefore play an essential part in the preparation of future generations of therapeutic biotechnological products. Their adoption will result in the simplication and improvement of downstream processing flow sheets and will enable a rapid transition between discovery and utilization of these products.

See also: **I/Affinity Separation:** Covalent Chromatography; Dye Ligands; Rational Design, Synthesis and Evaluation: Affinity Ligands; Theory and Development of Affinity Chromatography.

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Covalent Chromatography

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Introduction

Conventional affinity chromatography involves specific recognition of biomolecules such as antibodies and enzymes by immobilized ligands (antigens and inhibitors) usually by a multiplicity of non-covalent interactions. By contrast, the separation process in covalent chromatography does not require specific adsorptive binding and thus does not require knowledge of the structural determinants of the binding area of the component to be isolated. Instead, specificity relies on the nature of the chemical reaction of the chromatographic material with one or more components of a mixture. When complete specificity is

achieved in the bonding step, only one of the components reacts. The other components are removed by washing and the bonded component is then released by another chemical reaction. Ideally this leaves the chromatographic material in a form that is readily regenerated. When several components react with the chromatographic material, specific isolation of individual components needs to be achieved subsequently, e.g. in the elution step (sequential elution covalent chromatography). A recent extension of covalent chromatography involves derivatization specifically of thiol-containing components by reaction with a dithiopyridyl polyethyleneglycol (PEG) reagent. This provides charge shielding effects and facilitates separation of the derivatized proteins by ion exchange chromatography.

The development of covalent chromatography is discussed below and is summarized in **Table 1** in which key papers and reviews are identified. Those key papers not listed in the Further Reading section may be found in one or more of the reviews. Widespread application of the technique began after 1973, when covalent chromatography by thiol–disulfide interchange using the 2-mercaptopyridine leaving

Table 1 Milestones in the development of covalent chromatography and some key publications

1963	*Fundamental paper* reports the synthesis of an 'organomercurial polysaccharide' for the isolation of thiol-containing proteins and the first example of covalent chromatography (Eldjarn and Jellum).
1970	*Fundamental paper* reports unusual high reactivity of the thiol group of papain towards 2,2′-dipyridyl disulfide (2-Py-S-S-2-Py; 2PDS) at pH 4 which provided the basis for covalent chromatography by thiol–disulfide interchange with provision for selectivity for low pK_a thiol groups (Brocklehurst and Little).
1972	*Fundamental paper* reports an early example of covalent affinity chromatography (a combination of covalent and conventional affinity chromatographies) in which penicillin-binding proteins are isolated by reaction with the β-lactam ring of immobilized 6-aminopenicillamic acid and released by reaction with hydroxylamine (Blumberg and Strominger).
1973	*Fundamental paper* introduces covalent chromatography by thiol–disulfide interchange for the isolation of fully active papain using a Sepharose-(glutathione-2-pyridyl disulfide) gel (Brocklehurst, Carlsson, Kierstan and Crook; marketed by Pharmacia).
1975	*Fundamental paper* reports the synthesis and use of a more highly substituted gel with an electrically neutral and less sterically demanding spacer, the Sepharose 2-hydroxypropyl-2′-pyridyl disulfide gel (Axén, Drevin and Carlsson; marketed by Pharmacia).
1978	*Fundamental paper* reports the introduction of *N*-succinimidyl-3-(2′-pyridyl disulfanyl) propanoate which readily permits the introduction of auxiliary thiol groups into non-thiol-containing proteins to widen the scope of targets for reversible immobilization by thiol–disulfide interchange (Carlsson, Drevin and Axén).
1980	*Review* cites approx. 150 papers on covalent chromatography published between 1973 and 1978; although most publications are concerned with thiol-containing proteins, there are some references to covalent chromatography involving serine, methionine and tryptophan side chains and to the isolation of nucleic acids and membrane fragments (Lozinskii and Rogozhin).
1981	*Fundamental paper* reports development of sequential elution covalent chromatography to separate protein disulfide isomerase and glutathione insulin transhydrogenase (Hillson).
1982	*Review* discusses selectivity by proton-activated covalent chromatography using 2-pyridyl disulfide gels in acidic media as a logical extension of the more general use of soluble disulfides containing the 2-mercaptopyridine leaving group in protein chemistry and enzymology as enzyme active centre titrants, reactivity probes, delivery vehicles for spectroscopic reporter groups and heterobifunctional crosslinking reagents (Brocklehurst).
1983	*Fundamental paper* reports the use of $(Gly\text{-}Phe\text{-}Phe)_2$-cystamine immobilized on Affi-Gel10 (BioRad) for the isolation of cathepsin B; this is an example of an extension of the general method of covalent chromatography by thiol–disulfide interchange by provision of recognition sites to create a covalent affinity gel (Evans and Shaw).
1985	*Review* discusses covalent chromatography and its applications in biochemistry and biotechnology; extensive detailed descriptions are given of the synthesis, characteristics and commerical sources of activated support materials (Brocklehurst, Carlsson and Kierstan).
1995	*Fundamental paper* reports examples of selectivity in covalent chromatography by thiol–disulfide interchange determined by steric and electrostatic restrictions (Thomas, Verma, Boyd and Brocklehurst).
1996	*Review* summarizes applications of covalent chromatography by thiol–disulfide interchange with references also to the use of some other types of thiol-specific chromatography: organomercurials, isothiocyanates and 4-aminophenylarsenoxide–agarose for the selective isolation of molecules containing vicinal thiol groups (Brocklehurst).
1996	*Fundamental paper* discusses an example of a development of covalent chromatography whereby monomethoxypolyoxy-(ethylene glycol) (mPEG)-(glutaryl)-S-S-2-Py is used to derivatize components of a mixture of thiol-containing enzymes to facilitate their separation by ion exchange chromatography (Azarkan, Maes, Bouckaert, Thi, Wyns and Looze).

Table 2 Applications of covalent chromatography by thiol–disulfide interchange using 2-pyridyl disulfide-containing gels or 2-pyridyl disulfide derivatives of the target protein or peptide

Applications	*Comments*
Fractionation and specific isolation of thiol-containing proteins and peptides	Purification of a wide range of enzymes and other proteins by the various versions of the technique has been reported
Isolation and sequencing of thiol-containing peptides	Facilitates purification of thiol-containing peptides which is often difficult from proteolytic digests. Two versions: (i) immobilization of the protein by reaction with the disulfide gel followed by proteolysis; (ii) derivatization of the protein by reaction with 2PDS, proteolysis in solution and isolation by reaction with the thiolate gel
Removal of prematurely terminated peptides during solid-phase peptide synthesis	Premature chain termination of the peptide by blocking of the free terminal amino group results in unwanted by-products in solid-phase peptide synthesis. These are readily separated from non-terminated peptides by addition of Cys-Met to the free amino group of the non-terminated peptide prior to cleavage from the solid-phase matrix in preparation for covalent chromatography
Reversible immobilization of enzymes with associated purification	This method contrasts with most methods of immobilization which are irreversible. An additional advantage is that eventual release of the enzyme by thiolysis can produce purified enzyme if the preparation applied was not fully active
Synthesis of specific adsorbents for conventional affinity chromatography	Thiol–disulfide interchange provides a convenient method of attaching ligands containing specific recognition features to insoluble matrices

group was introduced by Brocklehurst *et al*., initially for the specific isolation of the fully active form of the cysteine proteinase, papain.

Various approaches developed subsequently are discussed including the range of gel types, the reactions involved in attachment, elution and gel reactivation and brief discussion of specific covalent attachment via groups other than thiol groups. The range of applications of covalent chromatography by thiol–disulfide interchange is summarized in **Table 2**.

Development of the Technique

Scope

Most of the published papers on covalent chromatography relate to proteins and peptides. The emphasis has been on thiol-containing molecules but immobilization procedures via the side chains of serine, methionine and tryptophan have been devised. In addition the use of covalent chromatography for the isolation of polynucleotides, low M_r co-factors, and gene fragments has been reported.

The Pre-Pyridyl Disulfide Era

The first example of covalent chromatography dates from 1963 when Eldjarn and Jellum reported the use of an organomercurial dextran based on Sephadex G-25 (**Figure 1**A) for the isolation of thiol-containing proteins which are released from the gel by treatment with low M_r mercaptans. Simpler and more effective products using a better support material (agarose) were developed subsequently, e.g. by Cuatrecasus

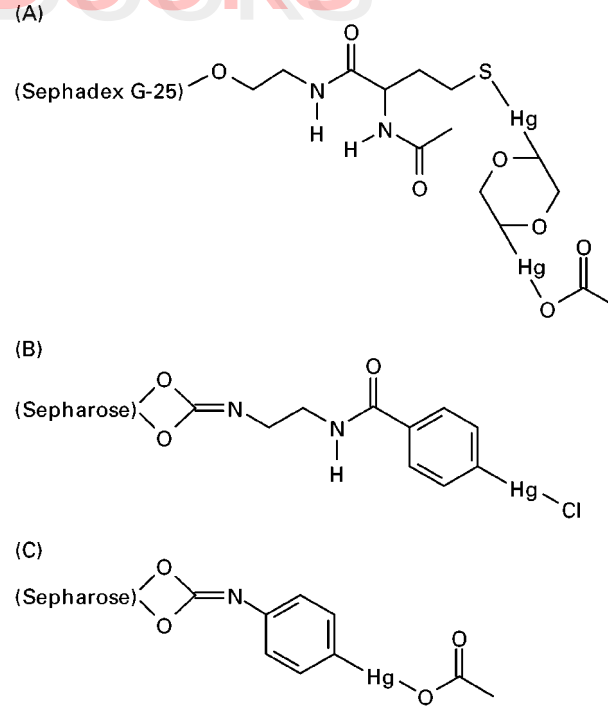

Figure 1 Some organomercurial gels. (A) Due to Eldjarn and Jellum, 1963. (B) Due to Cuatrecasus, 1970. (C) Due to Sluyterman and Wijdenes, 1970.

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(**Figure 1B**) and by Sluyterman and Wijdenes (**Figure 1C**) both in 1970. Some solid phase organomercurials are available commercially but as Lozinskii and Rogozhin pointed out in their review in 1980 (**Table 1**) relatively little interest was shown in the technique of covalent chromatography until the introduction of the version involving thiol–disulfide interchange using a solid phase 2-pyridyl disulfide gel in 1973 (**Table 1**). Problems with organomercurial gels include gradual loss of the metal with consequent contamination of the purified protein, lack of absolute specificity for thiol groups and lack of provision both for designed selectivity and of a means of spectral monitoring of occupancy of gel sites by the target protein.

Covalent Chromatography by Thiol–Disulfide Interchange Using 2-Pyridyl Disulfide Gels

The reactions involved in attachment, elution and gel reactivation Covalent chromatography using insoluble mixed disulfides containing the 2-mercaptopyridine leaving group (Gel-spacer-S-S-2-Py) was devised by Brocklehurst *et al.* in 1973 as a logical extension to the use of 2,2′-dipyridyl disulfide (2PDS or 2-Py-S-S-2-Py) as a thiol titrant with selectivity in acidic media for intact catalytic sites in the cysteine proteinase papain. In 1970 Brocklehurst and Little had observed unusually high reactivity of the thiol group of Cys25 in papain towards 2PDS which has its origin in the coexistence of the catalytic site ion pair motif, (Cys25)-S^-/(His159)-Im^+H, and the activated, protonated form of the disulfide, 2-Py-S-S-2-Py^+H. The coexistence of significant concentrations of these reactants arises from the low pK_a value for ion pair formation (3.3) and its relationship to the pK_a value of the 2-Py-S-S-2-Py^+H cation (2.45). Soluble reagents of the general type R-S-S-2-Py (reviewed by Brocklehurst in 1982; see Table 1) have proved useful in the study of thiol-containing proteins, e.g. as enzyme active centre titrants, reactivity probes, delivery vehicles for spectroscopic reporter groups and crosslinking reagents. 2,2′-Dipyridyl disulfide and simple alkyl-2-pyridyl disulfides successfully titrate intact catalytic sites in cysteine proteinases even in the presence of low M_r mercaptans or denatured enzyme that still retains its thiol group but with the ion pair disrupted. It was as part of a programme designed to exploit the two-protonic-state nature of reagents of the type R-S-S-2-Py^+H/R-S-S-2-Py where the protonated forms possess reactivities *c.* × 1000 greater than those of the unprotonated forms that covalent chromatography was originally devised. Thus selectivity of attachment in favour of low pK_a thiol groups may be achieved by carrying out the attachment procedure at pH *c.* 4 where reaction is with the protonated gel (Gel-spacer-S-S-2-Py^+H (see **Figure 2A**) and reaction of thiol groups with 'normal' pK_a values (8–10) will not occur because these will exist in the non-nucleophilic RSH forms. The technique is more generally applied in weakly alkaline media (pH 8) where reaction with the unprotonated gel (Gel-spacer-S-S-2-Py) (see **Figure 2B**) would be expected to occur readily with most thiol-containing compounds. Thus when covalent chromatography using a Sepharose-spacer-2-pyridyl disulfide gel is applied to the isolation of thiol-enzymes at pH 8, thiol-containing protein is freed from irreversibly oxidized and hence inactivated enzyme containing sulfinic acid ($-SO_2H$) groups in place of thiol groups. When applied, e.g. to cysteine proteinases at pH 4, attachment is specifically by reaction of the catalytically active form of the enzyme containing the essential (Cys)-S^-/(His)-Im^+H ion pair generated by protonic dissociation with pK_a of about 3.

Reaction of the thiol-containing protein with either protonation state of the gel may be quantified by spectral analysis of the chromophoric pyridine-2-thione released in the thiol–disulfide interchange (λ_{max} 343 nm, $\varepsilon_{343} = 8080\ M^{-1}\ cm^{-1}$). This provides a measure of the practical capacity of the gel for a particular protein. The theoretical capacity may be determined by reaction of the 2-pyridyl disulfide sites in the gel with a low molecular weight mercaptan such as 2-mercaptoethanol and spectral analysis of the pyridine-2-thione released into solution. The practical capacity is usually less than the theoretical capacity due to the inaccessibility of some sites to macromolecules. After removal of unreactive components by washing, the thiol-containing protein is released from the gel by elution with a reducing agent, usually a low molecular weight mercaptan (**Figure 2C**). During elution the gel is left in the non-activated, thiolated state (Gel-spacer-SH) and may be reactivated by reaction with 2PDS (**Figure 2D**).

An alternative version of this type of covalent chromatography involves derivatization of the thiol-containing protein (PSH) by reaction with 2PDS and attachment by reaction of P-S-S-2-Py so produced to a non-activated thiolated gel (**Figure 2E**). Other disulfide gels have been used subsequently for covalent chromatography, such as those prepared by reaction of thiol groups in gels with 5,5′-dithiobis-(2-nitrobenzoate). Such gels lack the ability to increase their reactivity by protonation at low pH and thus do not offer the possibility of selectivity that 2-pyridyl disulfide gels provide.

Support materials and spacers As in other separation techniques the solid support for the reactive

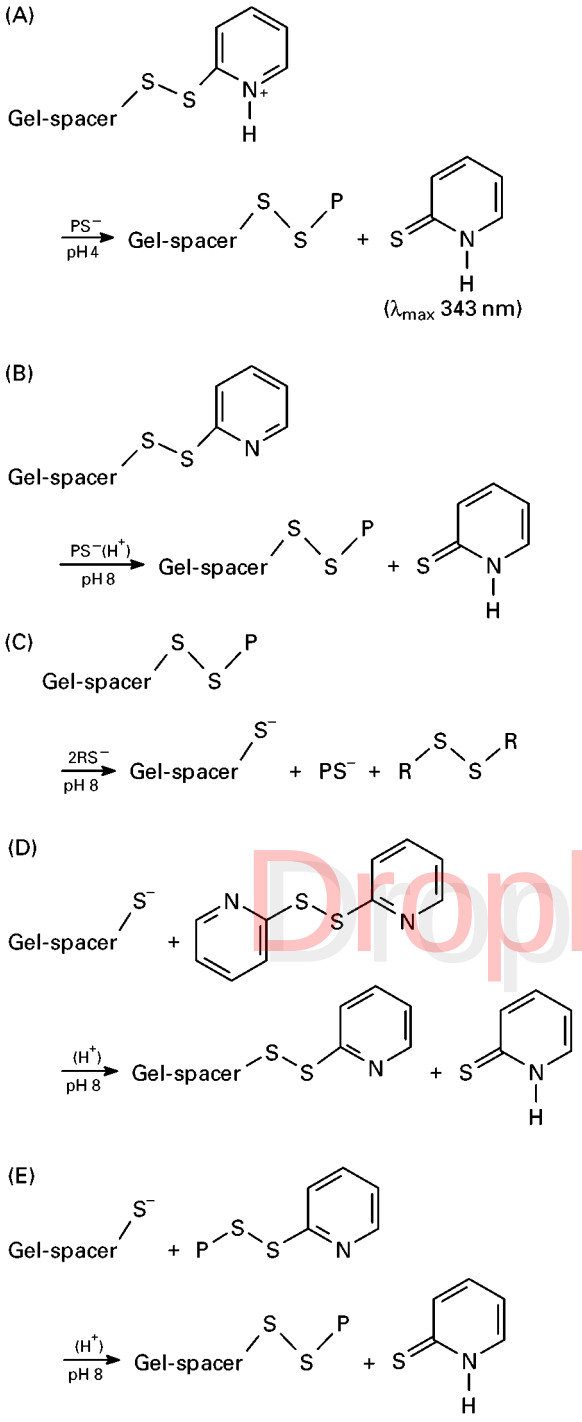

Figure 2 Reactions involved in covalent chromatography by thiol–disulfide interchange. (A) Selective attachment of a thiol-containing protein (PSH) containing a low pK_a thiol group by reaction with the protonated gel sites at pH values *c.* 4. (B) More general attachment of a thiol-containing protein containing a thiol group with a 'normal' pK_a value (8–10) by reaction with the unprotonated gel sites in weakly alkaline media (e.g. pH 8). (C) Elution of the thiol-containing protein by reaction with a low molecular weight mercaptan (RSH). (D) Reactivation of the thiolated gel by reaction with 2PDS. (E) Covalent chromatography using a non-activated thiolated gel and a protein-S-S-2-Py mixed disulfide prepared by reaction of the protein (PSH) with 2PDS.

groups in covalent chromatography must have sufficient mechanical, chemical and biological stability to resist degradation during the chromatographic process. It needs to be sufficiently permeable to permit access of macromolecules to reactive groups within the support material and sufficiently inert so as not to denature the molecules to be isolated. Spherical beads provide good column packing and flow properties. The support must allow opportunities to introduce the chemically reactive groups required for the immobilization process without serious perturbation of the other properties mentioned above. The support that has been most widely used in covalent chromatography is the polysaccharide, agarose. Beaded agarose is available, e.g. as Sepharose 2B, 4B and 6B and as crosslinked products with increased mechanical stability such as the CL-Sepharoses. The original (1973) version of covalent chromatography by thiol–disulfide interchange utilized the gel shown in **Figure 3A**, prepared by reaction of cyanogen-bromide-activated agarose with the amino group of glutathione followed by reaction of the thiol group with 2PDS. Use of the 5-nitro derivative of 2PDS provides an activated gel that releases a coloured thione (λ_{max} 386 nm) during the attachment of a thiol-protein. Spacers other than glutathione (e.g. cysteine, cysteamine and ethane) have been attached to agarose but the activated gel shown in **Figure 3B**, reported in 1975 by Axén *et al.* (**Table 1**) is particularly noteworthy. Whereas the glutathione gel (**Figure 3A**) is negatively charged, the hydroxypropyl gel (**Figure 3B**) is electrically neutral and, in addition, is more highly substituted and less sterically damanding. The difference in these characteristics accounts for the different selectivities exhibited by the two gels demonstrated in 1995 by Thomas *et al.* in connection with studies on the highly negatively charged enzyme actinidin and on chymopapain M, an enzyme that rejects all but the smallest ligands in one of its recognition sites. The activated glutathione gel bonds to all of the cysteine proteinases in *Carica papaya* except chymopapain M (for steric reasons) and fails to bond with actinidin because of electrostatic repulsions. More generally, the spacer between the gel and the reactive attachment site should not be long and hydrophobic in order to minimize non-specific hydrophobic effects. Neither should it possess substantial ion exchange properties. These requirements of course are common to any separation technique that relies on specific reaction or interaction with particular sites engineered into the gel. Other support materials that fulfil some or all of the requirements for a satisfactory chromatographic material include crosslinked polyacrylamide and inorganic materials such as porous

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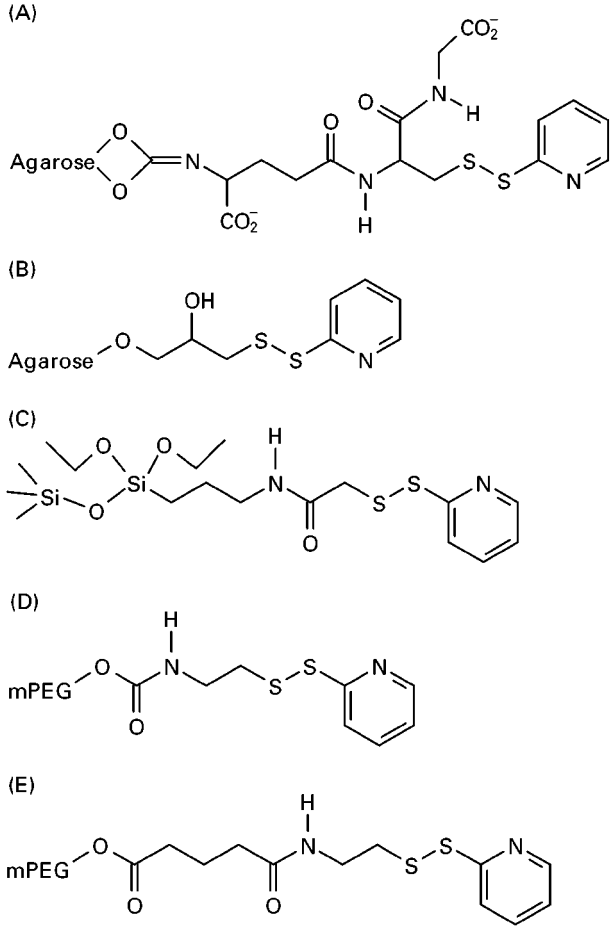

Figure 3 Some activated gels used in covalent chromatography by thiol–disulfide interchange. (A) The original (1973) Sepharose–glutathione-2-pyridyl disulfide gel. (B) The more highly substituted, electrically neutral, less sterically demanding (1975) Sepharose–hydroxypropyl-2-pyridyl disulfide gel. (C) A macroporous silicon oxide derivative (1979). (D) and (E) Two soluble mPEG derivatives used to modify the surface properties of thiol–enzymes by interactions with the monomethyoxypolyethylene glycol (mPEG) 5 kD chains (1995/96).

glass coated with hydrophilic polymers. An example of an inorganic material that has been used in covalent chromatography is the macroporous silicon oxide derivative (**Figure 3C**) reported by Lozinskii *et al*. in 1979.

Sequential elution covalent chromatography This extension to the technique was introduced by Hillson in 1981. A mixture containing different thiol-containing proteins is applied to a 2-pyridyl disulfide gel and in most cases all would be expected to react. Separation is achieved in this case in the elution step. Elution either with different concentrations of a given mercaptan or with a series of mercaptans each of different redox potential results in the sequential elution of each component with consequent separation.

Use of mPEG–Enzyme Mixed Disulfides in Conjunction with Ion Exchange Chromatography

During the mid-1990s Looze and co-workers introduced the use of soluble mixed disulfides containing the usual 2-mercaptopyridine leaving group and derivatives of monomethoxypolyethylene glycol (mPEG; nominal molecular mass 5 kDa) (**Figures 3D** and **3E**) for the isolation of thiol-containing enzymes by ion exchange chromatography. The chromatographic behaviour of the enzymes appears to be modified by the charge shielding effects of the PEG chain. This approach provides another means of separating components of mixtures of thiol–enzymes as an alternative to sequential elution covalent chromatography.

Other Types of Covalent Chromatography

Attachment via thiol groups Substitution of the 2-mercaptopyridine leaving group by other aromatic mercapto groups results in the loss of selectivity at low pH and does not appear to offer substantive advantage. The intramolecular agarose thiolsulfinates introduced by Carlsson and his colleagues in the mid-1990s provide an alternative to the mixed agarose–aromatic disulfide gels discussed above. Thiolated agarose is subjected to mild oxidation by potassium ferricyanide to produce disulfide groups followed by further oxidation to thiolsulfinate groups by a stoichiometric amount of magnesium monoperoxyphthalate. These gels also lack the opportunity to provide selectivity for low pK_a thiol groups at low pH. They do not require external leaving groups but because of that do not offer the possibility of measurement of reactive site content by thiolysis and spectral analysis. An example of covalent affinity chromatography using a substrate-like symmetrical disulfide (Gly-Phe-Phe-)$_2$-cystamine immobilized by amide bond formation on Affi-Gel 10 was reported by Evans and Shaw in 1983. In this type of approach specific binding interactions align the nucleophilic (thiolate) and electrophilic (disulfide) reactants for the covalent bonding process.

In some applications thiol groups exist as part of the support material and one example involving reaction of thiol–agarose gels with thiol–proteins derivatized as mixed disulfides by reaction with 2PDS (**Figure 2E**) constitutes one of the alternative versions of covalent chromatography by thiol–disulfide interchange. A different application of thiol–agarose gels is in studies on nucleic acids. Cytosine and uracil residues in polynucleotides can be mercurated

without appreciable change in function. These derivatives form mercaptides by reaction with thiol–agarose and are eluted subsequently by treatment with a low molecular weight mercaptan.

Attachment by reaction of thiol groups is not restricted to reaction at electrophilic sulfur. The higher reactivity of arylisothiocyanates towards thiol groups than towards amines permits their use in thiol-selective covalent chromatography. An immobilized tervalent organoarsenical, 4-aminophenylarsenoxide–agarose, has been used for the selective isolation of molecules and assemblies containing vicinal thiol groups (lipoic acid and the 2-oxoglutarate dehydrogenase multienzyme complex of which lipoic acid is a covalently bonded co-factor). Attachment involves cyclic dithioarsenite formation. Elution by 2,3-dimercaptopropane-1-sulfonic acid releases the reduced (dimercaptan) form of lipoic acid.

Attachment via seryl hydroxy groups Organophosphate agarose derivatives are an obvious choice for isolation of proteins with highly reactive seryl hydroxy groups such as the serine hydrolases. Coupling of 2-aminoethyl 4-nitrophenyl methyl phosphonate to succinylated aminoagarose produced a material that reacted specifically with serine hydrolases such as acetylcholine esterase and chymotrypsin. The problem with these gels is the very slow release of the enzymes even by good nucleophiles that provide reactivation in analogous soluble systems.

Attachment via methionyl thioether groups The known selectivity of alkylating agents for methionyl residues in acidic media to produce sulfonium derivatives and the possibility of regeneration by sulfur nucleophiles led Schechter *et al.* in 1977 to produce a chloroacetamidoethyl polyacrylamide derivative for the isolation of proteins via methionyl side chains. The relatively severe conditions required for attachment (low pH and long reaction times) limit the applications of this method. The methionyl residue cannot be at the C-terminus because such residues are converted to homoserine residues and attachment is not achieved. Regeneration of the covalent chromatography material is not provided for in this method.

Attachment via tryptophanyl side chains Arylsulfenyl chlorides and sulfur monochloride (S_2Cl_2) selectively modify tryptophan residues in acidic media to form 2-arylsulfenyl tryptophan and 2-mercaptotryptophan moieties respectively. Rubinstein *et al.* used this knowledge in 1976 to prepare polyacrylamide derivatives that react covalently with tryptophan-containing peptides which are released in modified forms by treatment with a low molecular weight mercaptan. The tryptophan side chain is converted to a 2-mercaptotryptophan side chain in the process. The method could find application in protein sequencing but is of limited use for protein isolation not only because of the necessary introduction of the mercapto group but also because of the requirement for acid stability of the protein.

Applications of Covalent Chromatography

The range of applications of covalent chromatography is illustrated in **Table 2** by reference to methods that utilize thiol–disulfide interchange. Examples of these applications can be found in one or more of the reviews listed in the Further Reading section.

Concluding Comments

The ease with which specificity and selectivity can be provided in covalent chromatography involving attachment via thiol groups, together with the advantages due to the mild conditions required for attachment, elution and reactivation of the gel, account for the outstanding success of versions of the technique involving thiol–disulfide interchange, particularly those using 2-pyridyl disulfide sites. Attachments via other protein side chains are generally less satisfactory in these respects and have been used only to a limited extent. Often covalent chromatography has been used at a relatively late stage in the purification process but the successful isolation of bovine mercaptalbumin from crude extracts reported by Carlsson and Svenson in 1974 suggests that this approach should be considered in other cases. Some of the applications listed in Table 2 can be applied to non-thiol-containing proteins by the introduction of an auxiliary thiol group, e.g. by use of the valuable heterobifunctional reagent, *N*-succinimidyl-3-(2′-pyridyl disulfanyl) propanoate, introduced by Carlsson, Drevin and Axén in 1978 or by site-directed mutagenesis.

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Dye Ligands

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Introduction

Dyes employed in protein and enzyme purification are synthetic hydrophilic molecules bearing a reactive, usually a chlorotriazine, moiety by which they can easily be attached to various polymeric supports. Among the various known reactive dye ligands, Cibacron Blue 3GA or F3GA (CB3GA, **Figure 1**A), an *ortho*-isomer of CI Reactive Blue 2, has attracted most attention from biotechnologists in protein purification. The foundations of the important role of CB3GA may well be attributed to pure historical accident; an anomalous gel permeation chromatography run of pyruvate kinase when using blue dextran as a void volume marker. It was later found that the blue chromophore, CB3GA, was responsible for binding the enzyme thus leading to co-elution of enzyme and blue dextran. As with many critical but unexpected discoveries, the importance and breadth of applications were hardly appreciated in those early days. Since then, CB3GA and other triazinyl dyes have been immobilized on to various supports and used in the affinity purification of many proteins and enzymes.

Development of Dye Ligands and Dye Affinity Adsorbents

The originally exploited dyes were commercial textile chlorotriazine aromatic polysulfonated molecules which when attached to appropriate supports, usually bearing hydroxyl groups, yield dye affinity adsorbents. The range of shades of commercial dyes derives primarily from anthraquinone, azo and phthalocyanine chromophores bonded to suitable reactive functions such as triazinyl and other mainly polyhalogenated heterocyclics. Anthraquinone dyes produce blue and the phthalocyanines turquoise shades. Green dyes contain mixed anthraquinone–stilbene, anthraquinone–azo or phthalocyanine–azo structures, whereas most other shades are derived from the azo class.

Unlike most biological affinity adsorbents, the stability of dye affinity adsorbents is usually limited only by the support itself. Dyes offer clear advantages over biological ligands, in terms of economy, ease of immobilization, safety, stability and adsorbent capacity. The main drawback of textile dyes is their moderate selectivity during the protein-binding process. In spite of this, the overall size, shape and distribution of ionic and hydrophobic groups enable dyes to interact with the binding sites(s) of proteins sometimes fairly specifically, as for example, with the nucleotide-binding site of several dehydrogenases, kinases, and several nucleotide-recognizing enzymes. The dye–protein interaction should not be compared to a simple ion exchange type since binding is frequently possible at pHs greater than the p*I* of the targeted protein. Furthermore, dissociation of the dye–protein complex is often achieved specifically by competing ligands, suggesting interaction with the protein at discrete sites. The view is supported by chromatographic, kinetic, inactivation, affinity labelling and spectra difference studies.

The last few years have seen a novel approach for tackling the problem of dye selectivity, signalling the